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Krüppel acts as a gap gene regulating expression of *hunchback* and *even-skipped* in the intermediate germ cricket *Gryllus bimaculatus*

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Abstract

In *Drosophila*, a long germ insect, segmentation occurs simultaneously across the entire body. In contrast, in short and intermediate germ insects, the anterior segments are specified during the blastoderm stage, while the remaining posterior segments are specified during later stages. In *Drosophila* embryos, the transcriptional factors coded by gap genes, such as *Krüppel*, diffuse in the syncytial environment and regulate the expression of other gap, pair-rule, and Hox genes. To understand the segmentation mechanisms in short and intermediate germ insects, we investigated the role of *Kr* ortholog (*Gb'Kr*) in the development of the intermediate germ insect *Gryllus bimaculatus*. We found that *Gb'Kr* is expressed in a gap pattern in the prospective thoracic region after cellularization of the embryo. To determine the function of *Gb'Kr* in segmentation, we analyzed knockdown phenotypes using RNA interference (RNAi). *Gb'Kr* RNAi depletion resulted in a gap phenotype in which the posterior of the first thoracic through seventh abdominal segments were deleted. Analysis of the expression patterns of Hox genes in *Gb'Kr* RNAi embryos indicated that regulatory relationships between Hox genes and *Kr* in *Gryllus* differ from those in *Oncopeltus*, another intermediate germ insect. Furthermore, we found that *Gb'Kr* regulates expression minimally of *hunchback* and *even-skipped*, directly or indirectly, in the prospective thoracic region. Our findings suggest that *Gb'Kr* is a gap gene that acts in the cellular environment and is required for segmentation in the thoracic and abdominal regions through the regulation of gap and pair-rule gene expression.

Keywords: *Gryllus bimaculatus*; Intermediate germ; Gap gene; *Krüppel*; RNAi; Segmentation

Introduction

Most insects develop as short or intermediate germ embryos, where only the anterior segments are specified almost simultaneously during the blastoderm stage. The remaining more posterior segments are formed later successively, from anterior to posterior. In contrast, long germ insects specify all segments simultaneously during the blastoderm stage. Since the short and intermediate germ modes are found in diverse and phylogenetically basal groups of insects, ancestral insect embryogenesis probably resembled short or intermediate germ embryogenesis (Davis and Patel, 2002; Tautz et al., 1994).

The molecular mechanisms of segmentation are well understood in the long germ insect *Drosophila melanogaster* (Pankratz and Jackle, 1990). Here, maternal gradients provide information to define positions along the anteroposterior axis of the syncytial embryo. Maternal gradients of Bicoid, Hunchback, and Caudal proteins, along with Torso activity in the terminal region, are required for activation of zygotic gap genes in broad domains. Gap gene products in turn diffuse and produce overlapping short-range gradients (Hülskamp and Tautz, 1991; Rivera-Pomar and Jäckle, 1996). These short-range gradients then serve to regulate the expression of primary pair-rule genes in stripes of a two-segment periodicity (Klingler and Tautz, 1999; Small and Levine, 1991). Gap gene products also provide positional information for the expression of Hox genes, which assign identities to the specified segments (McGinnis and Krumlauf, 1992).

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In short and intermediate germ insects, cellularization of the blastoderm occurs relatively early (Ho et al., 1997; Sarashina et al., 2005), so that the orthologs of gap genes in these insects often appear to be active in a cellularized environment (e.g. Bucher and Klingler, 2004; Liu and Kaufman, 2004; Mito et al., 2005). Thus, the gap gene orthologs of short and intermediate germ insects may act in a different manner from those in the *Drosophila* syncytial embryo. Thus, elucidating the functions of the gap genes in short or intermediate germ insects would provide crucial clues to clarify the molecular segmentation mechanisms in these insects.

The cricket, *Gryllus bimaculatus*, is an intermediate germ insect well suited for functional analysis by RNA interference (RNAi) (Miyawaki et al., 2004; Shinmyo et al., 2005; Mito et al., 2005). To date, a number of its segmentation genes have been examined. Orthologs of the segment polarity genes, *wingless* and *hedgehog*, are expressed in similar stripe patterns in *Gryllus* and *Drosophila*, suggesting that their functions in segment patterning are conserved (Niwa et al., 2000; Miyawaki et al., 2004). The functions of more upstream factors in *Gryllus* segmentation, however, apparently differ from those in *Drosophila* segmentation. The *Gryllus* ortholog of the *caudal* gene (*Gb'cad*) appears to play a more prominent role in anterior segmentation than in *Drosophila*, affecting the expression of the gap gene orthologs, *Gryllus hunchback* and *Krüppel* (Shinmyo et al., 2005). Additionally, unlike the situation in *Drosophila*, Wingless/Armadillo signaling has been shown to play a role in the posterior segmentation in *Gryllus* possibly through the regulation of *Gb'cad* expression (Miyawaki et al., 2004; Shinmyo et al., 2005). Furthermore, *Gryllus hunchback* has been shown to function in a non-canonical manner in segment patterning (Mito et al., 2005).

In the present study, we focused on *Krüppel* (*Kr*), a *Drosophila* gap gene. *Drosophila Kr* encodes a transcription factor that contains four zinc-finger motifs and is required for formation of central segments of the embryo (Gaul et al., 1989; Rosenberg et al., 1986). Null alleles of *Kr* result in a canonical gap phenotype that lacks the first thoracic through fourth abdominal segments, and the fifth abdominal segment is partially deleted (Gloor, 1950; Wieschaus et al., 1984). In the intermediate germ insect, *Oncopeltus fasciatus*, *Kr* has been also reported to act as a gap gene for formation of the second thoracic through fourth abdominal segments, despite divergent embryogenesis in *Oncopeltus* and *Drosophila* (Liu and Kaufman, 2004). However, in the short germ insect, *Tribolium castaneum*, *Kr* appears to function differently from *Drosophila* and *Oncopeltus Kr* because its mutation does not result in deletion in thoracic and anterior abdominal segments but display a homeotic transformation in these segments (Bucher et al., 2002; Sulston and Anderson, 1996). Thus, the function of *Kr* gene apparently varies among insect species.

To gain insights into the evolution of *Kr* functions and segmentation mechanisms in insects, we have investigated the roles of the *Kr* ortholog in *G. bimaculatus*, the most basal insect in which *Kr* function has yet been investigated. We examined its expression patterns during embryogenesis and analyzed its functions using RNAi depletion. We found that *Gryllus Kr* (*Gb'Kr*) is a gap gene that acts in the cellular environment and is required for segment patterning in the thoracic and abdominal regions through regulation minimally of the expression of *Gryllus hunchback* and *even-skipped*. Based on results of our RNAi analyses, we propose a model for the regulatory network between *Kr* and other segmentation genes in *Gryllus*.

Materials and methods

Whole-mount in situ hybridization

Standard protocols were used for whole-mount in situ hybridization with a digoxigenin (DIG)-labeled antisense RNA probe, as previously described (Niwa et al., 2000). In situ hybridization for double staining was done as previously described (Mito et al., 2005).

RNAi

We synthesized double-stranded RNA (dsRNA) for *Gb'Kr* and *DsRed2* using the MEGA-script Kit (Ambion) and used PCR fragments as the template for in vitro transcription as previously described (Miyawaki et al., 2004; Mito et al., 2005). The PCR fragments were amplified using upstream and downstream primers that contained the T7 promoter sequence. The final concentration of dsRNA was adjusted to 20 μM for the *Gb'Kr* dsRNA (267 bp, spanning the Type 1 to 3 zinc fingers) and the *DsRed2* dsRNA (660 bp, derived from the pDsRed2-N1 (Clontech)). The *DsRed2* dsRNA was used for negative control experiments. For parental RNAi, we injected a 0.6 μl dsRNA solution into beneath the joint of the coxa of the T3 leg of an adult female cricket. Fifteen injected females were mated with untreated males, and the eggs were collected from 5 to 10 days after injection.

Results

Expression patterns of Gryllus Krüppel during embryogenesis

We isolated a fragment of the gap gene ortholog *Gryllus Krüppel* (*Gb'Kr*), which encodes the Type 1 to 3 zinc fingers and part of the Type 4 zinc finger of the five zinc fingers (Types 1–5) in *Drosophila Kr* (Rosenberg et al., 1986; Mito et al., 2005). At 30 h after egg laying (hAEL), *Gb'Kr* is expressed in the posterior region of the germ anlage (Fig. 1A). At 32 hAEL, the *Gb'Kr* expression domain shows a band pattern with an anterior to posterior gradient in the posterior one-third of the embryo (Fig. 1B). At 38 hAEL, *Gb'Kr* is expressed in a gap-like domain in the middle region of the embryo (Fig. 1C). The *Gb'Kr* gap domain corresponds to the posterior labial segment through the third thoracic segment, as shown by double staining with *Gryllus wingless* (*Gb'wg*) (Fig. 1D). The gap domain of *Gb'hb* corresponds to the mandibular segment through the anterior of the labial segment (Mito et al., 2005). Thus, the gap domains of *Gb'Kr* and *Gb'hb* are adjacent. By 40 hAEL, *Gb'Kr* expression appears in the anterior head region (Fig. 1D), changing dynamically into a complex pattern (Fig. 1E). In the early germbands, *Gb'Kr* expression in the underlying mesoderm, in addition to the ectodermal expression, initiates in the gap domain and expands anteriorly, increasing its intensity (Figs. 1E, F). This anterior expansion of the mesodermal expression is finally covers the mandibular segment (Fig. 1G). During germband elongation, *Gb'Kr* is also expressed in the mesoderm of the extending posterior region in a segmentally reiterated pattern progressing from the anterior to the posterior (Fig. 1G). The *Kr* mesodermal expression seems to be conserved in other insect species ever examined (Gaul et al., 1987; Sommer and Tautz, 1991; Liu and Kaufman, 2004). After the germband finishes